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(12) UK Patent Application (19) GB (11) 2 275 774 (13) A**(43) Date of Publication 07.09.1994****(21) Application No 9403348.7****(22) Date of Filing 22.02.1994****(30) Priority Data****(31) 8302067****(32) 23.02.1993****(33) FR****(71) Applicant(s)****Pasteur Sanofi Diagnostics****(Incorporated in France)****3 Boulevard Raymond-Poincaré-8P3,
92430 Marnes La Coquette, France****(72) Inventor(s)****Catherine Larue****Pierre-Yves Marquet****(74) Agent and/or Address for Service****Frank B Dehn & Co****Imperial House, 15-19 Kingsway, LONDON,
WC2B 6UZ, United Kingdom****(51) INT CL⁵****G01N 33/96 33/68****(52) UK CL (Edition M)****G1B BAH B622 B639****C3H HK1****U1S S1332 S1337 S3009****(56) Documents Cited****None****(58) Field of Search****UK CL (Edition M) C3H HK1****INT CL⁵ G01N 33/53 33/68 33/96****Online databases:WPI; Biotech (dialog)****(54) Stabilized composition of troponin****(57) Stabilized composition of troponin I or T for immunoassays, comprising an aqueous solution or powdered preparation containing troponin I or troponin T, 1 to 10 molar equivalents of troponin C per molar equivalent of troponin I or T, and optionally Mg⁺⁺ and/or Ca⁺⁺ ions, T.****GB 2 275 774 A**

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The present invention relates to a stabilized composition of troponin used as standard in serum or blood plasma troponin immunoassays.

5 It is known that troponin is a myofibrillar protein complex consisting of 3 proteins, troponins C, I and T, which triggers the regulation of muscle contraction by Ca^{2+} , which contraction results from the interaction of myosin and actin of myofibrils.

10 When muscle is damaged, whether the cardiac muscle, during a myocardial infarction or the skeletal muscle, during prolonged physical exercise, the contractile proteins then released appear more or less rapidly in the blood stream.

15 Thus, the determination of troponins for the early diagnosis of myocardial infarction has recently been proposed, whether that of troponin T in Circulation 83 902-912 (1991) or of troponin I in Am. Heart J 110 1333-44 (1987) and Molecular Immunology 29 (2) 271-278 (1992).

20 Any enzymatic immunoassay or any radio-immunoassay used in pathology laboratories involves, in general, the supply by the manufacturer, in addition to the reagents required for the assay, that is to say labelled or non-labelled antibodies, tracer agents and
25 solutions for dilution, of a standard for the compound to be assayed which, when used under conditions similar to those for the sample to be studied, will serve as reference for calculating the results and/or as positive control.

30 It is known that proteins are not very stable in solution, and reagents containing them are frequently marketed in freeze-dried form, together with a solvent, of suitable composition, in which the said reagents will

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have to be dissolved by the user before use ; keeping the solutions obtained at 4°C makes it possible to use them for several days, even if daily calibration shows some variation in the concentrations of the reagent ; in general, and this is what is recommended for troponin T, the standard solutions prepared from the freeze-dried product are frozen, in unit dose.

Buffered aqueous solutions of troponin, especially those of troponin I and T, can be stored for several months at -80°C, but it has been observed that they are not stable for more than a few hours at +4°C, even if protease inhibitors or antibacterial agents were added thereto, thereby forcing pathology laboratories to frequently prepare, sometimes twice daily, their solutions for calibration.

The present invention allows storage, for several days at +4°C, of more or less diluted standard solutions of troponin I or troponin T which are used as reference in specific immunoassays.

The stabilized composition of the invention comprises, in aqueous medium, one of the two troponins I or T depending on the assay to be carried out and from 1 to 10 molar equivalents of troponin C and preferably from 2 to 5 equivalents, as well as a large amount of Mg⁺⁺ and/or Ca⁺⁺ ions. The Mg⁺⁺ and/or Ca⁺⁺ ions are present in the form of salts, particularly chloride, bromide or nitrate. The quantity of Mg⁺⁺ and/or Ca⁺⁺ salt, may be 100 to 10000 times by weight that of troponin I or troponin T.

Troponin C may be of human or animal origin.

The concentration of troponin I or T in the solutions according to the invention correspond to those generally used in immunoassays, as a rule between 0.01 ng/ml and 1 µg/ml and preferably between 0.2 ng/ml and 25 ng/ml, while the concentration of Mg⁺⁺ and/or Ca⁺⁺

salts, which is not critical, may be between 20 μ m and 10 mM ; conventionally, this concentration will be close to 2 mM.

5 The solution may be buffered, in a conventional manner, to a pH of between 4 and 10, preferably 5.5 and 7.5, and the solvent may consist, partially or totally, of normal human plasma, in order to have a standard sample comprising same components as the sample to be studied, which contains the plasma or the serum
10 from the patient.

The subject-matter of the invention is also a powdered troponin I or T composition, preferably in freeze-dried form, optionally comprising Mg^{++} and/or Ca^{++} ions, such as $CaCl_2$, and from 1 to 10 molar equivalents
15 of troponin C, although in this case the presence of troponin C will be useful for ensuring the stability of the other troponins only from the time when the composition will have been dissolved in an aqueous solution by the user.

20 Possibly, the freeze-dried composition does not contain Mg^{++} and/or Ca^{++} ions. In that case, they are introduced at the dissolution step.

The subject-matter of the invention is also a process for stabilizing a solution of troponin I or T
25 for immunoassays, consisting in adding from 1 to 10 molar equivalents of troponin C per molar equivalent of troponin I or T, and Mg^{++} and/or Ca^{++} ions, for example $CaCl_2$.

30 In what follows, exemplary embodiments of the invention and the corresponding preservation test results are described.

Human troponin I (TnI) was isolated from a heart by the method described in FEBS Lett. 40 253-257 (1974). The solution obtained can be preserved for
35 several months at $-80^{\circ}C$, at a concentration greater than

10 µg/ml in phosphate-buffered saline containing 0.5% casein.

Troponin T (TnT) can be obtained as described in J. Biochem. 72, 723-735 (1972) or in J. Biol. Chem. 249 4742-4748 (1974).

Troponin C (TnC) can be isolated by the method described in Acta Chem. Scand. B 42 ; 211-215 (1988) from the complex of the 3 troponins (T, C, I), of bovine origin, which is on the market.

The concentrations of the solutions prepared are determined with Bradford reagent, described in Ann. Biochem. 72 248 (1976), and which is marketed ; the standard product is a known mixture of troponin I, C and T, marketed by the company SIGMA, freeze-dried, with the reference T 4895.

Preparation of a composition according to the invention

- Composition containing 5 molecules of troponin C per molecule of troponin I.

276 µg of CaCl₂, 2H₂O, 10 µl of troponin I solution at 10 µg/ml and 50 µl of troponin C solution at 10 µg/ml are introduced into 940 µl of KH₂PO₄ buffer (0.1 M ; pH 6.8) containing 10% normal human plasma.

It is preferable to carry out these operations in sterile medium using troponin I and troponin C solutions sterilized for example by passing them through a filter with a pore diameter of 0.22 µm.

The solution obtained, having TnI concentration about 100 ng/ml and TnC concentration about 500 ng/ml, is then used to prepare a series of dilutions from 1 ng/ml to 10 ng/ml of troponin I.

- Solutions containing 1 or 2 or 10 molar equivalents of troponin C relative to troponin I are prepared in the same manner.

The powdered composition can be obtained by

freeze-drying an aqueous composition prepared as above but with human plasma.

Solutions containing 1 to 10 molar equivalents of troponin C relative to troponin T are prepared in a similar manner by substituting troponin I with troponin T.

Test procedure

The solutions stored at 4°C are assayed on the chosen days, over 6 weeks, using standard calibration series, prepared immediately before use, from a troponin I solution preserved at -80°C.

The assay is performed with two monoclonal antibodies directed against the myocardial troponin I ; the first antibody is adsorbed onto the walls of immunoassay tubes marketed by NUNC (USA) under the reference Maxisorp, Startube ; the second antibody is labelled with peroxidase ; the method is a sandwich method.

The preparation of these antibodies is described in Molecular Immunology 29 (2) 271-278 (1992). It was observed that there is no interference in this assay with the other isoforms of troponin I, troponin C or the other myocardial proteins.

In the absence of TnC, from the first day of storage, a substantial decrease in the TnI concentration is observed for all the dilutions ; if TnC is replaced with actomyosin or with tropomyosin, at the rate of 5 molar equivalents relative to TnI, the concentration of TnI measured after 12 days is now only 2/3 of that of the starting concentration of 10 ng/ml whereas the solutions stabilized with TnC and CaCl₂ are not altered.

After 40 days, the TnI solutions, stabilized with various concentrations of TnC and CaCl₂, are not altered, whereas the non-stabilized solutions have an apparent concentration which may fall by up to 80%.

CLAIMS

1. Stabilized composition of troponin I or troponin T for immunoassays, comprising an aqueous solution containing troponin I or troponin T, 1 to 10 molar equivalents of troponin C per molar equivalent of troponin I or T, and Mg^{++} and/or Ca^{++} ions.
2. Composition according to Claim 1, containing an amount of Mg^{++} and/or Ca^{++} salts, particularly $CaCl_2$, between 100 to 10000 times by weight of troponin I or troponin T.
3. Composition according to one of Claims 1 and 2, comprising troponin I and 2 to 5 molar equivalents of troponin C per molar equivalent of troponin I.
4. Composition according to one of Claims 1 to 3, wherein the dilution medium is an aqueous solution buffered to a pH of between 4 and 10.
5. Composition according to Claim 1, wherein the solvent consists of up to 100% human plasma.
6. Composition according to one of Claims 3 to 5, wherein the concentration of troponin I is between 0.01 ng/ml and 1 $\mu g/ml$ and in that the concentration of Ca^{++} salts is between 20 μM and 10 mM.
7. Powdered composition for the preparation of a stabilized composition according to one of the preceding claims, containing troponin I or troponin T, 1 to 10 molar equivalents of troponin C per molar equivalent of troponin I or T and optionally Mg^{++} and/or Ca^{++} ions.
8. Composition according to one of the preceding claims, containing $CaCl_2$.
9. Composition according to Claim 7 in freeze-dried form and free of Mg^{++} and/or Ca^{++} ions.
10. Process for stabilizing an aqueous composition of troponin I or T for immunoassays, consisting

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ting in adding from 1 to 10 molar equivalents of troponin C per molar equivalent of troponin I or T, and Mg^{++} and/or Ca^{++} ions.

- 5 11. Process according to claim 10, consisting in adding a quantity of Mg^{++} and/or Ca^{++} salts, particularly $CaCl_2$, preferably equal to 100 to 10000 times by weight of troponin I or troponin T.

Patents Act 1977**Examiner's report to the Comptroller under Section 17
(The Search report)****- 8 -****Application number
GB 9403348.7****Relevant Technical Fields****(i) UK Cl (Ed.M) C3H (HK1)****(ii) Int Cl (Ed.5) G01N 33/53, 33/68, 33/96****Search Examiner
NICOLA CURTIS****Date of completion of Search
26 MAY 1994****Databases (see below)****(i) UK Patent Office collections of GB, EP, WO and US patent specifications.****Documents considered relevant
following a search in respect of
Claims :-
1-11****(ii) ONLINE DATABASES: WPI, BIOTECH (DIALOG)****Categories of documents**

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Category	Identity of document and relevant passages	Relevant to claim(s)
	NONE	

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